Short Communication

Bleeding complications in dengue are not associated with significant changes in the modulators of the endothelial barrier

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Abstract

Bleeding complications in dengue may occur irrespective of the presence of plasma leakage. We compared plasma levels of modulators of the endothelial barrier among three dengue groups: bleedings without plasma leakage, dengue hemorrhagic fever, and non-complicated dengue. The aim was to evaluate whether the presence of subtle alterations in microvascular permeability could be detected in bleeding patients. Plasma levels of VEGF-A and its soluble receptors were not associated with the occurrence of bleeding in patients without plasma leakage. These results provide additional rationale for considering bleeding as a complication independent of endothelial barrier breakdown, as proposed by the 2009 WHO classification.

Key words: dengue; bleeding tendency; VEGF

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Introduction

In 2009, a new dengue classification was proposed by the World Health Organization (WHO) to improve the detection of potentially severe complications of this disease, the so-called "warning signs" [1]. However, one of the possible limitations for the implementation of this classification on a routine basis is the fact that the biological rationale supporting the categories utilized has not been fully elucidated.

Bleeding is a common complication of dengue [2]; it has been traditionally described along with plasma leakage due to increased vascular permeability, and thrombocytopenia, as a syndrome known as dengue hemorrhagic fever (DHF) [3,4]. In the revised WHO classification, bleeding manifestations appear as independent dengue complications, but the mechanisms of bleeding in patients without plasma leakage are not fully understood. Although thrombocytopenia and an acquired coagulopathy could explain these manifestations [5,6], it is not possible to rule out that patients without plasma leakage were presenting subtle alterations in endothelial barrier function that were not detected by methods used in the clinical arena.

Vascular endothelial growth factor (VEGF)-A is a molecule involved in angiogenesis and is a potent modulator of the endothelial barrier, likely by promoting the destabilization of intercellular junctions [7]. At least two of the VEGF-A receptors are expressed on endothelial cells, VEGFR-1 and VEGFR-2. The binding of VEGF-A to VEGFR-2 on the cell membrane leads to an increase in vascular permeability [8]. Besides the transmembrane form, both receptors also have soluble forms that are believed to act as decoy receptors. Soluble VEGFR-1 binds *in vitro* to VEGF-A, blocking its interaction with cell surface receptors [9], thus balancing its deleterious effects [10]. The function of the soluble receptor sVEGF-R2 is less known, but it is possible that it regulates VEGF-A signaling on endothelial cells by a similar mechanism [11].

The modulation of the endothelial barrier by VEGF-A and its soluble decoy receptor, sVEGFR-2, has been recently detected as a possible mechanism involved in the pathogenesis of DHF. It has been demonstrated *in vitro* that dengue virus could downregulate the expression of sVEGF-R2 by the endothelial cells, promoting an excess of free VEGF-A

and contributing to the occurrence of plasma leakage [12].

Therefore, the aim of this study was to evaluate if patients with bleeding manifestations, but without the classical clinical and laboratory signs of plasma leakage, may present pathological changes in modulators of the endothelial barrier similar to those found in DHF. The rationale for the study was to evaluate biological mechanisms that may discriminate dengue with bleeding complications from DHF, as clinically suggested by the 2009 WHO classification.

Methodology

The circulating levels of VEGF-A, sVEGFR-1, and sVEGFR-2 in patients with dengue were evaluated; the patients presenting with bleeding complications without plasma leakage were compared with those with bleeding and plasma leakage (classified as DHF) and with patients with non-complicated dengue.

Patients with suspected dengue infection were selected during distinct outbreaks of dengue in southeast Brazil, in the cities of Rio de Janeiro in 2008, and Campinas in 2010, in two different hospitals and three primary care medical centers. Dengue infection was confirmed when anti-dengue IgM tested positive, after the fifth day of fever onset.

The inclusion criteria were suspected dengue infection, age over 17 years, and being in the defervescence period. The defervescence period was detected according to the medical follow-up at the primary care medical centers or at the hospitals. Usually, patients were enrolled in the study on the day they were tested for dengue serology (after the fifth day of fever), according to the Brazilian Ministry of Health protocol. Patients who met the inclusion criteria were reported to the study personnel, who evaluated if the patients could be enrolled in the study. After enrollment, patients were followed up prospectively until recovery.

Exclusion criteria were chronic kidney or liver disease, autoimmune or chronic infectious disease, hematological disorders, and neoplasia. Pregnant women were excluded. Patients who did not follow up after enrollment were excluded. When the serology results were available, patients with negative anti-dengue IgM were excluded.

The serological tests for the detection of anti-dengue virus IgM and IgG antibodies were performed by enzyme-linked immunosorbent assay (ELISA) with commercial kits (Panbio Dengue, Alere, Massachusetts, USA), and were conducted at the public health referral laboratories. Patients with positive anti-IgM were diagnosed with dengue.

Blood samples for the study tests were collected on the day of enrollment in the study. The study was conducted in compliance with the Helsinki Declaration, and was approved by the Research Ethics Committee of the Faculty of Medical Sciences of the University of Campinas. Written informed consent was obtained from patients or their relatives before any study procedure. Plasma levels of VEGF and its soluble receptors, sVEGFR-1 and sVEGFR-2, were determined by commercially available ELISA assays (Quantikine, R&D Systems, Minneapolis, USA).

In addition, because sVEGFR-1 and -2 may modulate VEGF-A activity, the ratio between VEGF-A and its soluble receptors was also calculated, in order to obtain additional information about the activity of the VEGF-A pathway in the regulation of endothelial barrier integrity. The ratio between VEGF-A and its soluble receptors was calculated by dividing the plasma concentration of VEGF-A by the plasma concentration of the decoy receptors sVEGFR-1 and sVEGFR-2, as previously described [13].

To compare continuous variables among three or more groups, Kruskal-Wallis analysis was used, followed by the Tukey test to identify the differences, when necessary. Data were analyzed using SPSS for Windows version 10.0 (SPSS Inc, Chicago, IL, USA) and SAS System for Windows version 9.2 (SAS Institute Inc, Cary, NC, USA). Graphics were made using GraphPad Prism, version 4 for Windows (GraphPad Software Inc., La Jolla, CA, USA). $P < 0.05$ was considered statistically significant.

Results

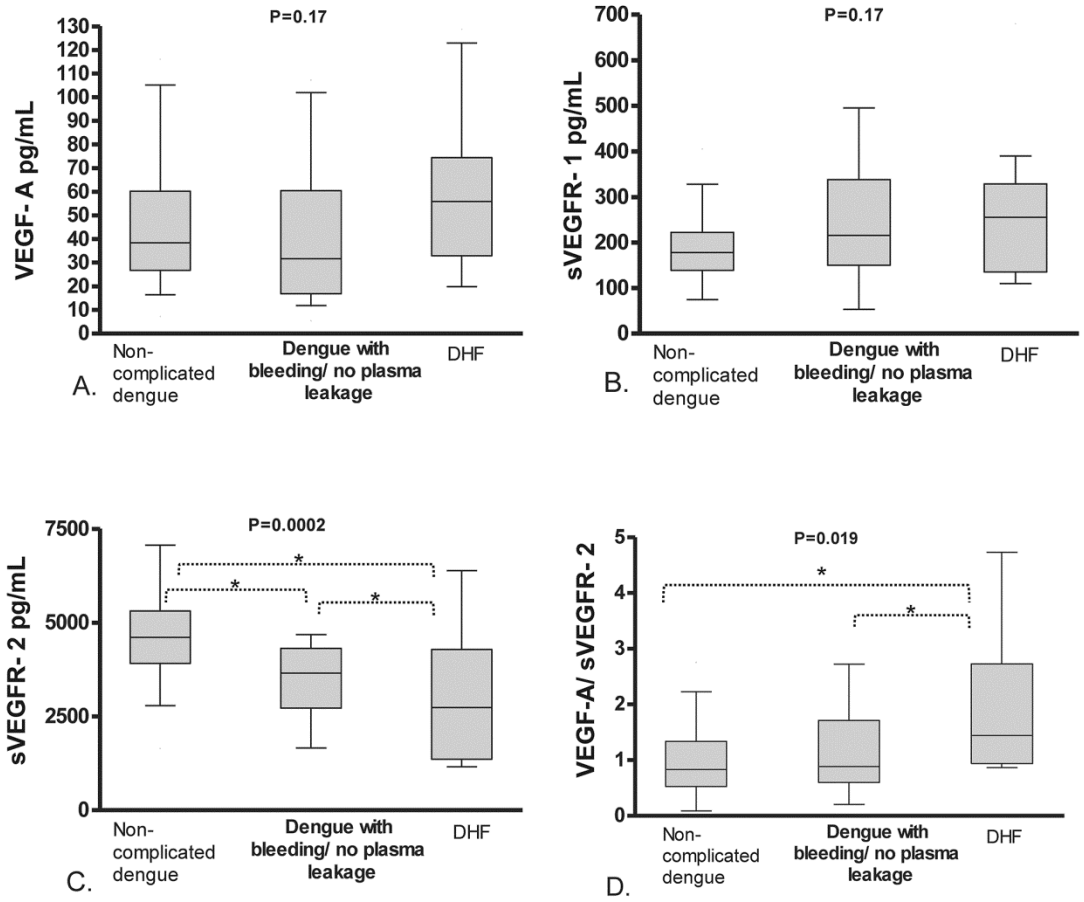
Three hundred and nineteen adult patients presented with suspected dengue infection in the period of the study; 63 patients were hospitalized because of complications, and 256 attended the primary care medical centers. From the 63 patients with complications who were hospitalized, 6 refused to participate, 14 had signs of convalescence (bleeding cessation and platelet arise), and 7 were anti-dengue IgM negative; these patients were excluded. From the 36 patients included, 10 were classified as having dengue hemorrhagic fever and 26 patients as having dengue fever and spontaneous bleeding, according to the traditional WHO classification [3]. From the 256 patients who attended the primary care centers, 178 patients were not enrolled in the study because they were in the febrile phase of dengue, did not collect anti-dengue serology, or lost the follow-up.

Table 1. Complete blood count and levels of microvascular permeability modulators

Parameters†	Dengue with bleeding/no plasma leakage (n = 26)	Non-complicated dengue (n = 33)	DHF (n = 10)	P
Days from the onset of fever	6.5 (1-14)	6 (3-10)	6 (3-11)	0.4
Hemoglobin, g/dL	13.6 (12.6-14.6)	14.7 (13.6-15.6)	13.8 (11.8-15.5)	0.12
Hematocrit, %	43.0 (37.0-45.0)	43.3 (40.6-46.3)	40.0 (36.0-50.0)	0.42
Leucocytes, /mm^3	5600 (4700-7660)	4040 (3385-6300)	6000 (4075-6540)	0.17
Platelets × 10^9/L	21 (14-36)	203 (126-283) *	17 (11-22)	**<0.0001**
VEGF-A pg/mL	31.9 (16.8 -60.4)	38.33 (26.8-60.3)	55.9 (32.9-74.4)	0.17
VEGFR-1 pg/mL	220.6 (150.5-338.3)	178.0 (140.1-221.3)	258.0 (135.6-328.8)	0.17
VEGFR-2 pg/mL	3,741(2,719-4,312)	4,609 (3,979-5,305)*	2,682 (1,357-4,287) *	**0.0002**
VEGF-A/VEGFR-1	0.13 (0.06-0.32)	0.23 (0.12-0.28)	0.23 (0.10-0.47)	0.26
VEGF-A/VEGFR-2	0.88 (0.2-5.55)	0.83 (0.09-2.75)	1.81 (0.86-10.9) *	**0.019**

†Median and interquartile range. Values represent the nadir hematocrit and platelet counts obtained during the defervescence.
P values were calculated using the Kruskal-Wallis analysis to compare the three groups of dengue patients, and were followed by the Tukey post-test.
*Place of difference in comparison with the group of dengue fever with bleeding but without plasma leakage detected by the Tukey post-test

Figure 1. Plasma levels of modulators of microvascular permeability.



Plasma levels of modulators of microvascular permeability in dengue patients. **A.** VEGF-A, **B.** sVEGFR-1, **C.** sVEGFR-2, **D.** VEGF-A/sVEGFR-2 ratio. P values were calculated using the Kruskal-Wallis analysis to compare the three groups of dengue patients, followed by the Tukey post-test to detect the place of differences. *Place of differences detected by the Tukey post-test.

One patient had chronic myeloid leukemia, one patient had a previous history of idiopathic thrombocytopenic purpura, three patients had chronic kidney disease, eighteen refused to participate, and five blood samples were handled inadequately; these were all excluded from the study. From the 50 patients who were included, 33 had positive anti-dengue IgM. All were classified as non-complicated dengue fever according to the traditional WHO classification [3].

Sixty-nine patients were included in the study; 26 patients had bleeding complications without plasma leakage, 33 had non-complicated dengue infection, and 10 had DHF. All patients were included around the sixth day after the onset of fever, during the defervescence period, as shown in Table 1.

Bleeding severity was similar between patients without plasma leakage and patients with DHF. Mild bleeding events, such as petechiae, ecchymosis, epistaxis, and gingivorrhagia occurred in 16/26 patients without plasma leakage (61.5%) and in 7/10 patients with DHF (70%). Moderate bleeding events, such as hemoptysis, hematemesis, hypermenorrhea, hematuria, and melena occurred in 10/26 patients without plasma leakage (38.5%) and in 3/10 patients with DHF (30%). The results of blood counts and modulators of microvascular permeability are shown in Table 1. Modulators of microvascular permeability are also illustrated in Figure 1.

As demonstrated in Table 1, patients with bleeding complications without plasma leakage did not present significant changes in VEGF-A or sVEGFR-1 levels. The only significant change observed in these patients was a lower level of sVEGFR-2 compared to non-bleeding patients, as shown by the Tukey post-test. However, the VEGF-A/sVEGFR-2 ratio was not altered in this group of patients. On the other hand, a shift towards the activation of the VEGF-A pathway in DHF could be observed by a non-significant increase in plasma VEGF-A levels, coupled with a more than twofold increase in the VEGF-A/sVEGFR-2 ratio ($p = 0.019$) in these patients compared to the others.

Discussion

In the present study, dengue patients with bleeding complications were evaluated for the presence of changes in modulators of the endothelial barrier. Given that the bleeding manifestations in patients with dengue are commonly accompanied by increased vascular permeability and plasma leakage, we hypothesized that more subtle changes in microvascular permeability, not detected by the classical clinical and laboratory evaluations, could be present in bleeding patients without plasma leakage, evidencing that these patients suffer a pathological mechanism similar to what is suggested in patients with DHF. The discrimination of bleeding and plasma leakage as two distinct dengue complications, as proposed by the 2009 WHO classification, may not be adequate for diagnosis and treatment purposes if both complications are biologically similar.

Increased VEGF-A levels have been described in patients with conditions associated with disruption of the endothelial barrier, such as septic shock [14]. In the context of dengue, it has been demonstrated that dengue virus could down-regulate the secretion of sVEGFR-2 by endothelial cells, thus promoting an increase in microvascular permeability via excess of circulating VEGF-A [15,16]. Indeed, clinical studies have demonstrated that patients with DHF may present high levels of VEGF-A [17] and low levels of sVEGFR-2 [15,18]. Accordingly, it has been suggested that plasma leakage occurring in this context could also contribute to occurrences of bleeding [19].

In our study, similar to that found in the previous reports cited above, higher VEGF-A levels were observed in patients with DHF, and a more than twofold higher VEGF-A/sVEGFR-2 ratio was observed in patients with DHF compared to the other groups. Dengue patients without plasma leakage did not present significant changes in plasma levels of VEGF-A and its soluble receptors, suggesting that the bleeding complications in these patients were indeed not associated with increased vascular permeability. To our knowledge, the role of the modulators of the endothelial barrier has not previously been evaluated in non-dengue hemorrhagic fever syndromes.

Therefore, according to our results, plasma levels of modulators of the endothelial barrier may not be associated with the occurrence of bleeding in patients with dengue without signs of plasma leakage, suggesting that, different from what was found in DHF, disruption of the endothelial barrier is not involved in the pathogenesis of bleeding manifestations in these patients.

It is important, also, to point out that the sample size may have influenced the results. The small sample size is justified because patients were selected in a late phase of the disease and many lost the follow-up before the time of enrollment. However, this study did not have epidemiological purposes, and the sample size had enough power to show the differences in modulators of endothelial barrier bleeding patients,

with and without plasma leakage, because these differences were very significant.

These results may confirm that bleeding is a dengue complication independent of changes in endothelial barrier function, as proposed by the new WHO classification [1]. While raising additional questions about the mechanisms of bleeding in these patients, these results support the implementation of the new dengue classification proposed by the WHO.

Conclusion

Bleeding complications in dengue infections (other than DHF) may not be associated with increased vascular permeability detected by circulating levels of modulators of the endothelial barrier.

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